RESEARCH COMMUNICATION

Mitochondrial proton conductance and H^+/O ratio are independent of electron transport rate in isolated hepatocytes

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In this paper we examine the non-linearity of the relationship between the proton electrochemical gradient across the mitochondrial inner membrane (Δp) and oxygen consumption of non-phosphorylating mitochondria *in situ* in hepatocytes. Models proposing to explain the non-linear relationship were tested experimentally. It was shown that the mitochondrial proton conductance and the number of protons pumped to the cytosolic side of the mitochondrial inner membrane by the electron transport complexes per oxygen atom consumed (H^+/O ratio) are independent of electron transport rate in mitochondria in isolated hepatocytes. The non-linearity of the plot of Δp against the non-phosphorylating oxygen consumption is due to either a potential-dependent slippage of the proton pumps of the mitochondrial inner membrane and/or a potential-dependent leakage of protons back across the mitochondrial inner membrane.

INTRODUCTION

The oxygen consumption by non-phosphorylating mitochondria titrated with a respiratory inhibitor shows a non-linear dependence on protonmotive force. This dependence has been observed for isolated mitochondria [1–8] and for mitochondria respiring in cells [9–13]. There are presently four models to explain this non-linearity. The first model [1] postulates increased proton leak conductance of the inner membrane at high proton electrochemical gradient across the mitochondrial inner membrane (Δp), while the number of protons pumped by the respiratory chain per oxygen consumed (H^+/O) remains constant. The second model [2] proposes decreased H^+/O pump stoichiometry as Δp increases, while the proton conductance of the inner membrane remains constant. This process is termed redox slip. The third model [5,6] proposes increased conductivity of the inner membrane to protons at high electron-transfer rates. In this model it is suggested that when the complexes are more active the inner membrane is more permeable to protons. Finally, the fourth model [3] proposes that there is increased redox slip at high turnover rates of the chain complexes.

Our laboratory has recently shown that the non-linearity of oxygen consumption by non-phosphorylating isolated mitochondria is solely due to increased proton conductance of the inner membrane at high membrane potential. That is to say, only model 1 is consistent with data for isolated mitochondria at 37 °C under the conditions investigated [14,15]. This result was also recently confirmed by Canton et al. [16] using rat liver mitochondria incubated under the same conditions, although there was evidence for redox slip at lower, unphysiological temperatures. However, it is still not clear whether model 1 holds true for mitochondria under physiological conditions. It is important to note that ion cycling *in situ*, leading to net proton leak, will be occurring across the inner membrane of mitochondria, e.g. Ca^{2+} cycling and proton flux due to transhydrogenase activity and NADPH re-oxidation, although the proportion of total mitochondrial leak *in situ* due to these coupled proton leak pathways has yet to be measured.

In this paper we examine the non-linearity of the relationship between Δp and oxygen consumption of non-phosphorylating mitochondria *in situ* in hepatocytes and show that it is caused by potential-dependent proton leak or redox slip.

EXPERIMENTAL

Preparation of hepatocytes

Rat hepatocytes were prepared essentially according to the method of Seglen [17]. Livers were perfused *in situ*. The calcium-free medium (medium I) contained (in mM): 118 NaCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 4.75 KCl, 25 $NaHCO_3$, 5 Hepes and 0.5 EGTA. The calcium-containing medium (medium II) was composed of (in mM) 118 NaCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 4.75 KCl, 25 $NaHCO_3$, 5 Hepes and 2.5 $CaCl_2$. Both media were constantly gassed with oxygen and carbon dioxide (O_2:CO_2, 95%:5%, v/v) to maintain oxygenation and constant pH. Collagenase, 20 mg/100 ml of medium II, was added and the perfusion medium was recirculated at 15 ml/min. No additions of heparin, trypsin inhibitors, defatted BSA or other digestive enzymes were made to either medium. Visibly digested liver, as a result of collagenase activity, was seen within 15 min. Subsequent stages in hepatocyte preparation were as described by Nobes et al. [10]. Cells were suspended in medium II and kept on

Abbreviations used: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; $\Delta\Psi_m$, membrane potential across the mitochondrial inner membrane; ΔJ_o, the difference in cellular oxygen consumption rate in the presence and absence of FCCP; Δp, proton electrochemical gradient across the mitochondrial inner membrane; ΔpH, pH gradient across the mitochondrial inner membrane; H^+/O, number of protons pumped to the cytosolic side of the mitochondrial inner membrane by the electron transport complexes per oxygen atom consumed; TPMP, methyltriphenylphosphonium; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

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ice before experimentation. Hepatocyte viability was routinely determined by exclusion of Trypan Blue (0.6 g/100 ml) in NaCl (0.89 g/100 ml). Only experimental results from hepatocyte preparations with viabilities greater than or equal to 90 % were used in this study.

Incubation of cell suspensions

Incubations of cell suspensions were carried out using 2.125 ml of incubation medium plus 0.375 ml of cell suspension (approx. 25 mg dry mass/ml) in 20 ml stoppered glass vials at 37 °C in a shaking water bath (100 cycles/min). The incubation medium contained (in mM) 106 NaCl, 5 KCl, 25 $NaHCO_3$, 0.41 $MgSO_4$, 10 Na_2HPO_4, 2.5 $CaCl_2$, 10 glucose, 10 lactate, 1 pyruvate and 2.25 % (w/v) defatted BSA. Stock 9 % (w/v) BSA was defatted by the method of Chen [18] and dialysed against 153 mM NaCl and 11 mM KCl. The gas phase above the incubating cells was 95 % air/5 % CO_2 to allow equilibration of the medium to a pH of 7.4. Titrations were initiated by the addition of 1 μg of oligomycin/ml incubation medium and 0, 0.05, 0.1, 0.5 or 1 μM myxothiazol. Titrations were performed in the absence of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) and at two submaximal concentrations: 1.5 and 3 μM. Cells were incubated for 10 min before the addition of inhibitor(s) and for a further 30 min in the presence of the inhibitor(s).

Calculation of mitochondrial membrane potential ($\Delta\Psi_m$)

$\Delta\Psi_m$ was calculated using eqn. (1):

$$\Delta\Psi_m = -61.5 \log\left\{\frac{V_c a_m}{V_m a_c}\left[\frac{[Cl^-]_{tot}\,[TPMP]_{tot}\,a_c(V_c+V_m)}{[Cl^-]_e\,[TPMP]_e\,a_e V_c}-1\right]\right\} \quad (1)$$

For an accurate value, the total accumulation of methyltriphenylphosphonium (TPMP) ($[TPMP^+]_{tot}/[TPMP^+]_e$) into the cell has to be corrected for factors, other than $\Delta\Psi_m$, that influence its accumulation. Details for the measurement of these parameters are given in Nobes et al. [10]. The ratio of mitochondrial to non-mitochondrial cell volume (V_m/V_c) was measured and was found to be 0.28 ± 0.06 ($n = 3$). The mitochondrial TPMP-binding correction (a_m, 0.44) was from Hafner et al. [4]. The cytoplasmic TPMP-binding correction (a_c, 0.21) was from Nobes et al. [10] and the value of $[Cl^-]_{tot}/[Cl^-]_e$ was measured and was found to be $0.58 \pm 0.01 (n = 3)$ for all titration conditions. The TPMP-binding correction for the 2.25 % (w/v) defatted BSA (a_e, 0.71) was from Nobes et al. [10]. The subscripts e, c, m and tot refer to extracellular, cytoplasmic plus nuclear, mitochondrial and total intracellular pools respectively.

Because proton leak is a function of Δp and not just $\Delta\Psi$, it is assumed in the titrations herein that ΔpH did not change disproportionately to $\Delta\Psi$ and therefore changes in $\Delta\Psi$ due to the titration conditions reflect the changes in the absolute value of Δp. On that point, ΔpH in hepatocytes is thought to be small [19] and Nobes et al. [10] have shown that at the concentrations of myxothiazol and oligomycin used in this study, ΔpH is probably not affected.

Materials

Oligomycin, valinomycin, BSA (fraction V), collagenase (type IV), EGTA, lactic acid, pyruvic acid and inulin were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.) TPMP-iodide and FCCP were obtained from Aldrich Chemical Co. (Gillingham, Dorset, U.K.). Potassium chloride, potassium phosphate, sodium bicarbonate, D-glucose and Hepes were from Fisons Chemical Equipment (Loughborough, U.K.). Magnesium sulphate, potassium bicarbonate and calcium chloride were obtained from British Drug Houses (Poole, Dorset, U.K.). Sodium chloride was obtained from Prolabo Group, Rhône-Poulenc Ltd. (Manchester, U.K.) Myxothiazol was from Boehringer Mannheim (East Sussex, U.K.). $Na^{36}Cl$ (86.6 μCi/ml), ^3H_2O and $^{86}RbCl$ (1 mCi/ml) were from Amersham International (Amersham, U.K.). [^{14}C]Methoxyinulin (11.8 mCi/g) and [^{3}H]TPMP-bromide (35 Ci/mmol) were from New England Nuclear (Du Pont de Nemours) (Bad Homburg, Germany).

THEORY

Let us first discuss discrimination between potential-dependent leak of protons across the mitochondrial inner membrane (model 1) and potential-dependent slip or redox slip (model 2). If we assume that a classical uncoupler of oxidative phosphorylation catalyses proton conductance ($C^{uncoupler}$) across the mitochondrial inner membrane in a Δp-independent manner, then the proton flux catalysed by this uncoupler has a linear dependence on Δp. That is to say, there is an ohmic relationship between proton flux and Δp for the classical uncoupler. This ohmic relationship has been observed for the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) [15,20]. Results in this paper demonstrate that FCCP also has this ohmic relationship.

If we now introduce a submaximal concentration of the classical uncoupler, in our case FCCP, to *in situ* mitochondria inhibited from making ATP, then the extra oxygen being consumed at each value of Δp in the titration must be used to drive protons through the uncoupler. If it is found that the relationship between this extra oxygen-consumption rate and Δp is linear, then H^+/O does not vary with Δp, implying that the original relationship between J_o and Δp in the absence of uncoupler is due to the Δp-dependence of the natural proton leak (model 1). If it is found that the relationship between ΔJ_o and Δp is non-linear, then the H^+/O ratio must decrease at higher Δp and the non-linearity of the original relationship between J_o and Δp must be due to Δp-dependent slip (model 2).

In algebraic terms the explanation is as follows. The extra proton influx will be given by C^{FCCP} times the value of Δp and will be equal to the extra proton efflux in the steady state. This in turn will be given by the extra oxygen consumption rate ΔJ_o multiplied by H^+/O. Therefore:

$$\Delta J_o = \frac{\Delta p \cdot C^{FCCP}}{H^+/O} \quad (2)$$

Dividing both sides of eqn. (2) by the concentration of FCCP added gives:

$$\frac{\Delta J_o}{FCCP} = \frac{\Delta p \cdot C^{FCCP}}{H^+/O \cdot FCCP} = \frac{\Delta p \cdot \text{constant}}{H^+/O} \quad (3)$$

A graph of $\Delta J_o/FCCP$ against Δp should pass through the origin and should have a slope inversely proportional to H^+/O. If H^+/O remains constant as Δp is varied (in other words, if the original non-linear titrations of Δp versus J_o are explained entirely by Δp-dependent changes in proton conductances (model 1), then this derived plot will give a straight line through the origin. If however, H^+/O decreases with increasing Δp, that is to say slip occurs (model 2), then the derived plot will give a curve with slope rising as Δp increases.

We can also test for proton leak and redox slip that depend on electron transfer rate (models 3 and 4) by using more than one concentration of FCCP. If leak depends on J_o (model 3) then the total proton conductance will be higher than anticipated when

more FCCP is present, and the graphs of ΔJ_o/FCCP against Δp for higher concentrations of FCCP will be steeper than those for lower concentrations. If slip depends on J_o (model 4), then H^+/O will be lower at higher FCCP concentrations, and again the graphs will be steeper. On the other hand, if models 3 and 4 are wrong and electron-transport rate does not influence the endogenous proton leak rate or H^+/O ratio, then the lines with different FCCP concentrations will superimpose on the plot ΔJ_o/FCCP against Δp.

Finally, the same experiment also tests for any direct effect of added FCCP; if FCCP itself changes the endogenous leak or causes slip, then once again higher FCCP concentrations will give steeper lines.

RESULTS AND DISCUSSION

Non-phosphorylating mitochondrial oxygen consumption (oxygen consumption in the presence sufficient oligomycin to inhibit mitochondrial ATP synthesis) was titrated with the respiratory chain inhibitor myxothiazol [21] in the absence of FCCP and at two submaximal FCCP concentrations (Figure 1). The non-ohmic nature of the titrations of oxygen consumption as a function of $\Delta\Psi_m$ in intact cells has been observed repeatedly before [9–13], and suggests that the oxygen consumption represents *in situ* leak or slip processes.

A secondary plot of ΔJ_o/FCCP against $\Delta\Psi_m$ (Figure 2) gave ten points which were fitted to a straight line using linear regression analysis ($P < 0.01$). The line intersects the x-axis at approximately 104 mV. The intercept may be due to ΔpH, which was not measured. Such intercepts are also seen with isolated mitochondria when using TPMP and are thought to be artefactual [14]. The points for the two different FCCP concentrations appear to superimpose, implying that there is no increase in endogenous proton conductance of the mitochondrial inner membrane and no decrease in H^+/O ratio as the turnover rate of the proton pumps increases, thus providing no support for model 3 or model 4. The linearity of the experimental data seen in Figure 2 is consistent with the assumption that the conductance catalysed by FCCP is ohmic, like the conductance catalysed by CCCP [14,15]. The superimposability of the data at each FCCP concentration (Figure 2) also confirms our prediction from the isolated mitochondrial experiments [14,15], that FCCP does not increase the endogenous permeability of the mitochondrial inner membrane to protons nor does it cause redox slip.

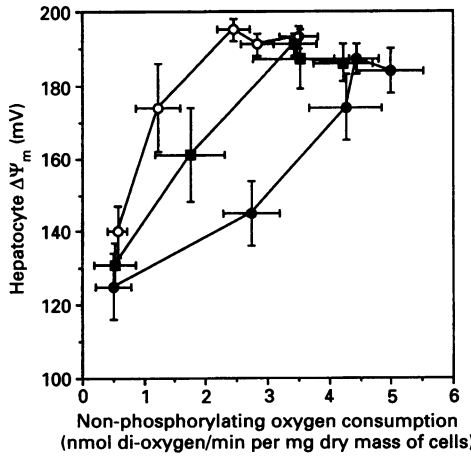

Figure 1 Myxothiazol titrations of non-phosphorylating hepatocyte mitochondria *in situ* in the presence of different submaximal concentrations of FCCP

Rat hepatocytes (25 mg dry mass/ml) were incubated in the presence of oligomycin (1 μg/ml) to inhibit mitochondrial ATP synthesis under conditions described in the Experimental section. Oxygen consumption was measured and titrated with myxothiazol (0, 0.05, 0.1, 0.5 or 1.0 μM) in the presence of 0 (○), 1.5 (■) or 3 μM (●) FCCP. The non-mitochondrial oxygen consumption [oxygen consumption in the presence of excess (3 μM) myxothiazol to inhibit electron transport] has been subtracted from the total oxygen consumption to give oxygen consumption due solely to mitochondrial processes.

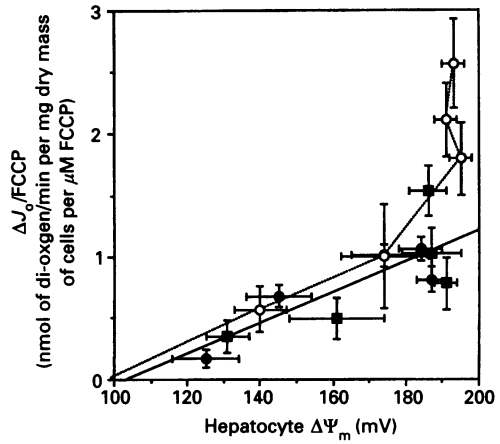

Figure 2 ΔJ_o/FCCP as a function of Δp

ΔJ_o is the difference between the measured value of J_o with FCCP present and the interpolated value at the same Δp in the absence of FCCP from Figure 1. FCCP concentration: (■) 1.5; (●) 3.0 μM. The continuous line is a linear regression calculated using all ten points with FCCP present; lines for the different FCCP concentrations were not significantly different from each other, thus failing to provide support for models 3 and 4. The dotted line (joining the open symbols) shows the result expected for these curves with FCCP present if the non-linearity of the non-phosphorylating mitochondria is caused entirely by potential-dependent redox slip (model 2). In order to generate these points we assumed that the lowest two points (at 140 and 174 mV) of the non-phosphorylating mitochondrial titration in Figure 1 have an ohmic relationship with Δp, and calculated the equivalent concentration of FCCP required for that ohmic relationship for the *in situ* mitochondria, which theoretically had zero endogenous leak; this value was 1.3 μM FCCP. The rest of the broken line was generated assuming zero endogenous leak, but an FCCP concentration of 1.3 μM.

The fact that the ten measured data points (closed symbols in Figure 2) fit a straight line, indicates that the non-linearity of the data in Figure 1 is due to an increase in the proton conductance of the mitochondrial inner membrane with increasing membrane potential, thus endorsing model 1. However, at high membrane potential these points (closed symbols) do not lie off the predicted broken line (Figure 2) for slip. The dotted line joining the open circles is the curve that would be expected if the non-ohmicity of the data in Figure 1 was due solely to slipping of the proton pumps at high membrane potential. Therefore, the data do not convincingly distinguish between redox slip and an increase in proton conductance at high membrane potential.

We conclude that the mitochondrial proton conductance and the H^+/O ratio are independent of electron transport rate in mitochondria in isolated hepatocytes. Therefore, the non-linearity of the plot of Δp against the non-phosphorylating oxygen consumption must be due to either a potential-dependent slippage of the proton pumps of the mitochondrial inner membrane and/or leakage of protons back across the mitochondrial inner membrane. However, we are unable to distinguish conclusively between these two mechanisms.

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